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Cysteine Residues in Thioredoxin

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Cysteine at 36th residue of thioredoxin from *Escherichia coli* was replaced with glycine by site-directed mutagenesis. Thioredoxin lost its original function as a protondonor to ribonucleoside diphosphate. The mutagenesis, however, did not affect thioredoxin's other function at all, i.e. interaction with gene 5 protein forming T7 DNA ploymerase.

Introduction

Thioredoxin of *Escherichia coli* shows two functions. Reduction of disulfide bond to thiol groups at 33rd and 36th cysteine residues coupled with oxidation of deoxyribonucleoside diphosphate(dDNP) being catalyzed by thioredoxin reductase(1). Its other function is observed when *E. coli* is infected with a bacteriophage T7. Gene 5 protein, which is encoded by T7 genome(2), complexes with a host protein, thioredoxin, to form T7 DNA polymerase(3). Gene 5 protein shows only exonuclease activity towards single-stranded (ss) DNA(4), unless thioredoxin is added. This latter function is referred to as Tsn C activity(3). The question as to what would happen if site-directed mutagenesis introduced a substitution of thioredoxin at the Cys residue is of interest. Would thioredoxin still be capable of making up a dimer with gene 5 protein thereby inducing DNA polymerase activity? This paper describes the DNA polymerase activity of gene 5 protein complexed with thioredoxin in which Cys-36 is replaced with Gly by site-directed mutagenesis.

Materials and Methods

Materials: M13 phage DNA mST20 containing *E. coli* thioredoxin was kindly made available by Dr. Richardson, Harvard University, USA. Oligonucleotide primer for mutagenesis, with the sequence of 3'-TGGTGCGGTCCGGGCAAAAT-5', was the gift from Dr. Nakamura, Osaka University. Bam HI, large fragment of DNA polymerase, T4 DNA polymerase, T4 polynucleotide kinase(PNK), bacterial alkaline phosphatase(BAP) and T4 DNA ligase were obtained from Takara Shuzo Co. or Toyobo Co. DNA sequencing

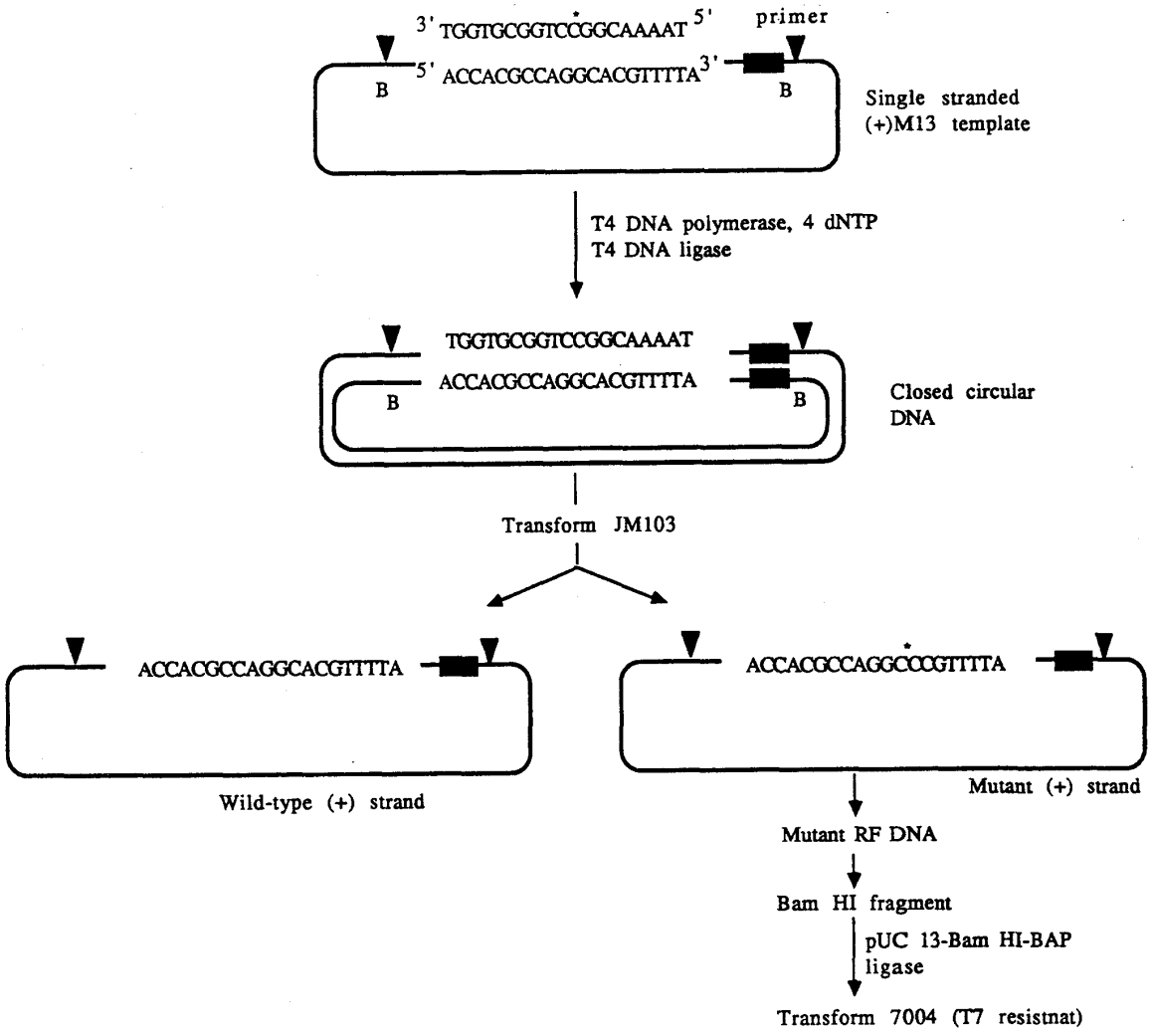

Fig.1 Strategy for site-directed mutagenesis (single primer method). Template: M13 mp9 DNA inserted with antisense sequences of thioredoxin (anti-mST20), B: Bam HI site, : insertion site, *: mutant point

was carried out by the dideoxy method(5,6) using a Takara sequencing kit. Nitro-cellulose filter used for dot hybridization was obtained from Schleicher and Schul, Co. Used as host strains were *Escherichia coli* JM103 and 7004.

Anti-mST20 preparation: 480-base pair(bp) thioredoxin gene was excised from mST20 replicative form(RP) and reacted with M13 mp9RF which was treated in advance with Bam HI and BAP, in the presence of T4 DNA ligase. After transfection of Ca^{++}-treated JM103 with the thus ligated sample, phage DNAs prepared from the resultant plaques were hybridized with mST20. The hybrid was then subjected to 0.8% agarose electrophoresis, which distinguished two types of DNAs: those which showed retardation while running and those which did not. The "retard" type DNA is designated as anti-mST20, and that is inserted in mp9 in the direction opposite to the positive strand. Above preparations were performed by the usual methods(7).

Labeling of primer at 5'-end: 98 pmole of primer was incubated with 10 μCi of 1 mM γ-[^{32}P] ATP and 5 units of PNK at 37 °C for 2 hours, according to the method of Maxam and Gilbert(8). The primer was then heated at 70 °C for 5 min.

Gene 5 protein preparation: T7-infected *E. coli* 7004 trxA, the mutant that produces incomplete thioredoxin, which is therefore T7-resistant, was disrupted by sonication and subjected to streptomycin and ammonium sulfate fractionation. Gene 5 protein was partially purified by DEAE-cellulose chromatography, as reported by Hori et al.(9) This preparation contains no DNA polymerase activity of the host cell.

Enzyme assays: The Tsn C activity of thioredoxin was determined by complementation with gene 5 protein, the object of which was to restore T7 DNA polymerase activity. The assay measures the incorporation of ^{3}H-TTP into acid-insoluble fraction. Exonuclease activity towards double-stranded (ds) DNA, shown after recon-

stitution of gene 5 protein with thioredoxin, was also assayed. Both methods were reported previously(10).

Site-directed mutagenesis: This was carried out by the so-called single primer method according to Winter et al.(11) as shown in Figure 1. Anti-mST20 (1 μg) was mixed, at 60 °C for 20 min, with 5 μg of 5'-phosphorylated primer (unlabeled, 50 times excess to template at molar ratio) in a solution of 0.1M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 0.26M NaCl, 5 mM dithiothreitol, and annealed in 40 min. This hybrid formation with a single mismatch was an initiation of polymerization of the new strand catalyzed by DNA polymerase. Addition of 1 mM ATP and 1 mM concentrations of dCTP, dATP, dGTP and TTP together with 5 units of T4 DNA polymerase, at 37 °C for 5 min, yielded two complmentary strands, which were ligated overnight at 16 °C at both ends by 150 units of T4 DNA ligase. Figure 2 shows 0.8% agarose electrophoresis of the ligated sample. Bands 1 and 2 were isolated by electroelusion in a dialysis bag at 80V for 20 min, extracted twice with phenol and precipitated with 2 vol of ethanol. Each DNA thus isolated was used for transfection of JM103 by the method of Mandel and Higa(12).

Preparation and identification of mutant phage DNA: 2 ml of culture of JM103 was shaken for 30 min, whereupon a phage plaque which emerged by transfection was added to JM103 using a toothpick, and the mixture was cultured for 7 hours. After centrifugation in an Eppendorf tube for 5 min, 1 ml of 30% polyethylene glycol-2.5M NaCl was added to the supernatant and made to stand at room temperature for 15 min. The mixture was then centrifuged for 10 min, and the precipitate was extracted with phenol, which was followed by ethanol precipitation. Each DNA was dissolved in 50 μl of water.

There would be a considerable amount of wild-type anti-mST20 in plaques (about 90%), which prompted dot hybridization

for identification of the mutant phage DNAs. The procedure was done according to a manual of Maniatis et al.(7)

Transformation of E. coli 7004 with plasmid containing mutagenized thioredoxin gene: A phage containing mutant DNA was inserted into JM103 and cultured for 7 hours or more. After collection of cells, RF DNA was obtained by the same method as in plasmid preparation. The RF was digested with Bam HI, and 480 bp fragment obtained by 0.8% agarose electrophoresis was ligated with Bam HI- and BAP-treated pUC13 in the presence of T4 DNA ligase. This sample was used for transformation of *E. coli* 7004. As a result, white colonies appeared on a agar plate containing 50 μg/ml ampicillin, IPTG and X-gal (inducer and substrate for beta-galactosidase).

Results and Discussion

As shown in Figure 1, the template DNA hybridizing with the primer should be an antisense strand in this case, because the primer sequences are the same as those of the positive strand of thioredoxin gene. Our M13 phage DNA named mST20 is an ss-DNA which is inserted with positive thioredoxin sequences at the Bam HI site. That was precisely why anti-mST20 was prepared in the first place.

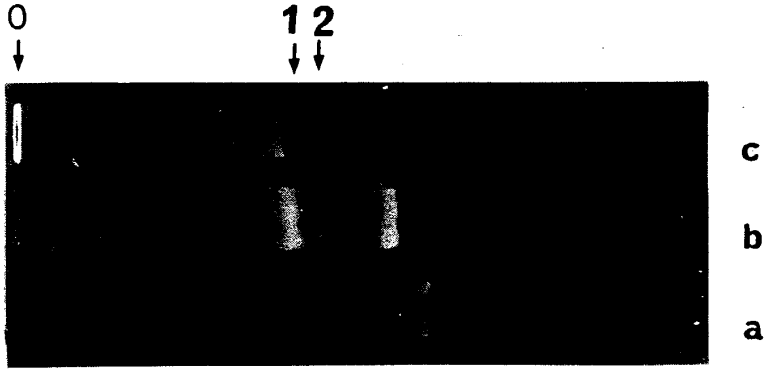

Fig.2 0.8% agarose gel electrophoresis. Lane a: anti-mST20, lane b: mutagenized sample, lane c: M13 mp9 RF DNA with no insertion

There are several improved procedures for site-directed mutagenesis(13-19). The method employed here is not as efficient as those widely accepted now. Ours is the cheapest method, however, because dot blot hybridization would be successful without requiring any special reagent. In dot hybridization, it is somewhat difficult to determine the precise condition under which wild-type and mutant DNAs are clearly distinguished. In Figure 3, spots with arrows are darker than others; darker spots represent mutant M13 DNAs, and lighter spots wild DNAs each with a single mismatch with the primer. Sequencing ascertained that the mutant DNAs contained desired nucleotide sequences in

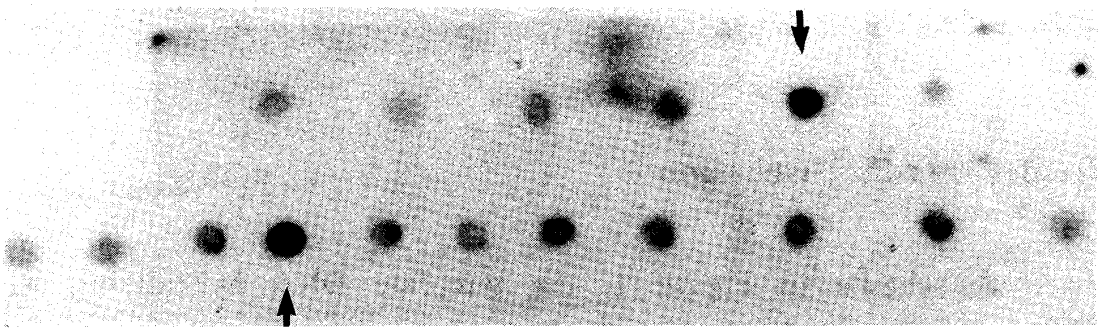

Fig.3 Dot blot hybridization of phage DNA. Nitro-cellulose filter with dotted DNAs was baked at 80°C for 3 hours. Prehybridization (3 hr) and hybridization (overnight) were done at 56 °C, and the filter was washed with 2 × SSC for 5 min, 3 times, at room temp., with 0.2 × SSC at 56°C for 1 hr, twice, and autoradiographed. SSC: dilute from 20 × SSC (3M Nacl-0.3M sodium citrate, pH 7.0)

strain	sequence	Amp	T7 titer (pfu/ml)	DNA polymerase(%)	ds DNA exonuclease(%)
B/1	-^{33}Cys-Gly-Pro-^{36}Cys	s	6.2×10^{10}	100	100
7004	———	s	0	1	3
pUCT1	-^{33}Cys-Gly-Pro-Cys	r	8.7×10^{10}	103	110
N25	- Cys-Gly-Pro-Gly-	r	8.1×10^{10}	98	80

Table 1. Properties of transformed strains and their Tsn C activities. s (r): sensitive (resistant) to ampicillin, pfu: plaque forming unit. Tsn C activities were expressed as % of specific activities of wild B/1.

which Gly was substituted for Cys at the 36th residue, as shown in Figure 4. Mutant phage DNAs were found from both band 1 and band 2.

We have examined whether transformed *E. coli* 7004 produced the type of thioredoxin which had Tsn C activity. The following became evident: i) plasmids from this strain acquired sequences for the mutant thioredoxin (data not shown); ii) this mutant proved to be sensitive to phage T7, while its original strain was T7-resistant; iii) thioredoxin which was partially purified by DEAE-cellulose chromatography exhibited DNA polymerase activity as well as exonuclease activity towards double-strand(ds) DNA, when combined with gene 5 protein. Table 1 summarizes the results just mentioned and includes activities of the wild-type thioredoxin, which was also inserted in pUC13 as control.

E. coli N25, which produces mutant thioredoxin, shows as much a T7 titer as that of *E. coli* pUTC1 where wild thioredoxin is present, and slightly more than wild strain *E. coli* B/1. Specific activities of these thioredoxins associated with gene 5 protein surely indicate that the presence of Cys at 36th residue is not a necessary condition for this protein to show Tsn C activity. Indeed, both DNA polymerase and exonuclease activities of the mutant thioredoxin are as high as those of the wild type. It is obvious that the mutant thioredoxin is not involved in the oxidoreduction pathway of rNDP at all, since there exists no thiol group to oxidize. This mutation is not lethal: as was the case in *E. coli* 7004, other kinds of reductants such as ascorbic acid would be used instead of thioredoxin in *E. coli* N25.

The single primer method mentioned here is laborious: large amounts of phage DNAs are needed for dot hybridization, and the primer must be purified perfectly for the maximum efficiency. It was precisely for this reason that another experiment

of ours on site-directed mutagenesis met with failure. In this study, we chose to employ T4 DNA polymerase in synthesis of a new strand, as shown in Figure 1. For those interested, it might be mentioned that a large fragment of *E coli* DNA polymerase I is also available. Site-directed mutagenesis will help in a further analysis of the mechanism of Tsn C activity of thioredoxin. As an example, Tabor and Richardson(20) improved the DNA sequencing method by employing chemically modified T4 DNA polymerase and this improved method is accepted widely. We are now in a position where we can produce a mutant thioredoxin, through substitution of one or more residues, which shows DNA

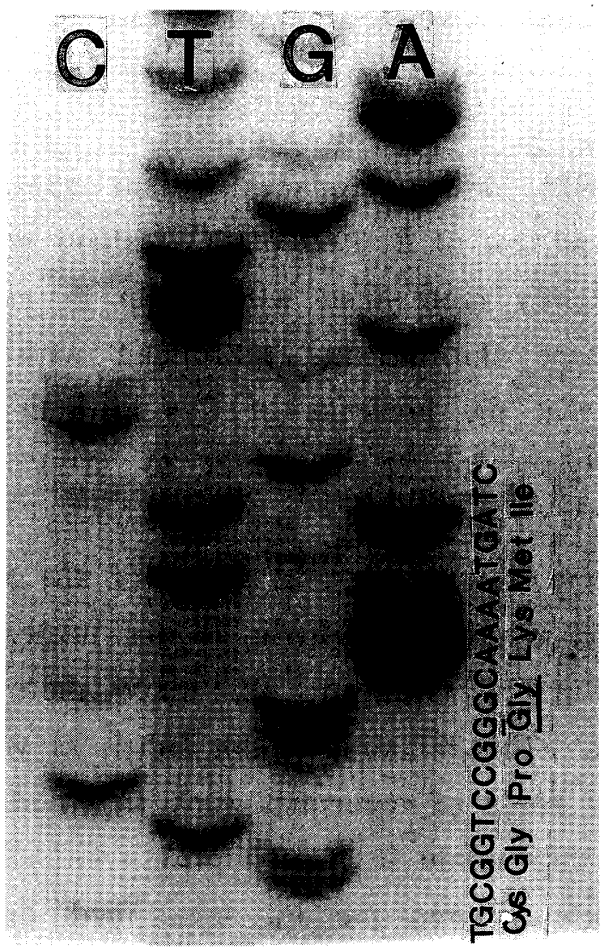

Fig.4 Sequencing of mutant phage DNA in 8% polyacrylamide-urea gel. Nucleotide sequences at the mutagenized region and corresponding amino acids were shown on right side.

polymerase activity but not exonuclease activity towards ds-DNA when added to gene 5 protein. Such a mutant would be useful not only in DNA technology but also in the study of reaction mechanism of DNA polymerase.

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チオレドキシンにおけるシステイン残基

高 崎 洋 三

大腸菌のチオレドキシンはヌクレオシド－２－リン酸（γＮＤＰ）の還元にカップルしてその２つのＣｙｓ残基を酸化してジスルフィド結合を形成するタンパク質である。

チオレドキシンはまたファージＴ７のＤＮＡポリメラーゼのサブユニットとしても働き，Ｔ７に由来する gene 5 protein はチオレドキシン共存下でないとポリメラーゼ活性を示さない。今回 site－directed mutagenesis の手法を用いて36番目のＣｙｓをＧｌｙに置換したもののポリメラーゼ活性について報告する。この置換により，チオレドキシンはγＮＤＰの還元について機能しなくなる訳だが，ＤＮＡポリメラーゼ活性には全く影響を与えなかった。同時に，２本鎖ＤＮＡに対するエキソヌクレアーゼ活性を調べたところ，これも野生型と同程度であった。この結果，チオレドキシンのＣｙｓ残基はＴ７ＤＮＡポリメラーゼ活性には関与していないことが分った。